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Immobilized Glucose-6-Phosphate Dehydrogenase as a Substrate for Solubilized Epidermal Growth Factor Receptor Tyrosine Kinase

A Convenient Microtiter Plate Assay System

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ABSTRACT

Based on our previously reported solution assay protocol, a solid-phase assay for the tyrosine kinase activity of the epidermal growth factor receptor has been developed. Glucose-6-phosphate dehydrogenase, immobilized noncovalently on microtiter plates, was used as the substrate in the solid-phase assay. Phosphorylation of the immobilized substrate takes place in the presence of ATP and a solubilized epidermal growth factor receptor preparation. After washing off the soluble reaction mixture, the phosphotyrosine-containing dehydrogenase produced on the well surface is quantitated by an ELISA method using a polyclonal antiphosphotyrosine antibody, a second antibody conjugated with horseradish peroxidase, and finally the *o*-phenylenediamine reaction. The absorbance at 492 nm developed in the wells is a measure of the kinase activity of the solubilized receptor preparation. Putative inhibitors of receptor kinase can be conveniently incorporated in this assay system to test for potential inhibitory activity. This assay, being rapid and convenient, is useful in drug screening programs where a high through-put rate is required.

Index Entries: Glucose-6-phosphate dehydrogenase; epidermal growth factor receptor; tyrosine kinase; antiphosphotyrosine antibody.

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INTRODUCTION

The receptor for epidermal growth factor (EGF) is a glycoprotein with a molecular wt of 170 kDa. The protein component of the receptor consists of a single polypeptide chain that transverses the plasma membrane once. The N-terminal portion of the receptor constitutes the ligand-binding domain.The cytoplasmic domain possesses specific tyrosine kinase activity (EC 2.7.1.112) that is stimulated by EGF binding to the extracellular domain (*1*). Kinase activity is not peculiar to the EGF receptor alone, but is in fact a common property of many growth factor receptors, some oncogene products, and their normal cellular homologs (*2*). The stimulation of the tyrosine kinase activity leading to increased levels of phosphotyrosine-containing proteins has been suggested to be physiologically important in the stimulation of cell growth and in the transformation of cells by certain viral oncogenes. Inhibitors of tyrosine kinase have thus become targets of intense research with a view to developing drugs capable of regulating cell differentiation and tumor transformation (*3*).

Proteins of intracellular origin have been identified as substrates for these tyrosine kinases, including lipocortin-like molecules (*4*) and glycolytic enzymes (*5*). In our previous study using a solution assay protocol, EGF receptor isolated from cultured human A431 cells has been demonstrated to phosphorylate certain tyrosine residues of glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), the rate-limiting enzyme of the pentose phosphate pathway (*6*). In succinct terms, the solution assay procedure involves: incubation of the solubilized EGF receptor with dissolved G6PDH and [γ-^{32}P] ATP, analysis of the phosphorylated proteins by sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE), localization of the G6PDH band by protein staining and autoradiography, and finally Cerenkov counting of the excised G6PDH band from the dried gel (*6*). A typical run of this procedure takes 2 d, and the number of lanes in a standard SDS-PAGE setup is limited. This severely restricts the applicability of this assay protocol to situations where a large number of assays have to be performed.

On the basis of our solution assay, a rapid and high-capacity microtiter plate assay was developed. This article describes in detail the design of this convenient assay system.

MATERIALS AND METHODS

Materials

A431 cells were obtained from American Type Culture Collection (Rockville, MD). Cell Culture supplies were from Flow (Costa Mesa, CA). Wheat germ agglutinin-Sepharose CL-4B was from Pharmacia (Uppsala,

Sweden). Na^{125}I was from Amersham (Buckinghamshire, UK). Iodogen, 1-ethyl-3-(3-dimethylaminopropyl) carbodimide, and keyhole limpet hemocyanin (KLH) were from Pierce (Rockford, IL). Bovine serum albumin (BSA), phosphoamino acids, Freund's adjuvants, G6PDH (Baker's yeast, crystalline), EGF (receptor grade), *o*-phenylenediamine, polylysines, random polymers, quercetin, and genistein were from Sigma (St. Louis, MO). Goat antirabbit immunoglobulin conjugated with horseradish peroxidase (HRP) was from Tago (Burlingame, CA). Other chemicals were of the highest quality available obtained from Merck (Darmstadt, Germany) or Sigma.

Source of EGF Receptor Tyrosine Kinase

Human epidermoid carcinoma A431 cells were grown at 37°C and 10% CO_2 atmosphere to confluence in a medium consisting of Dulbecco's Modified Eagle's Medium/Ham's F12 (50/50, v/v), 5% fetal calf serum, 15 m*M* HEPES, pH 7.2, 50 U/mL penicillin, and 10 μg/mL streptomycin. The cells were solubilized in 1% Triton X-100, and the EGF receptor was partially purified by wheat germ agglutinin-Sepharose affinity chromatography (*6*). Elution of the receptor was achieved with 40 m*M* HEPES, pH 7.5, containing 0.3*M* *N*-acetylglucosamine, 0.15*M* NaCl, 0.2% Triton X-100, and 10% glycerol. Protein concentration was determined by the method of Bradford (*7*). The affinity (approx 10^{-8}*M*) of the solubilized receptor for EGF and the receptor concentration (approx 5 pmol/mg protein) were determined by Scatchard analysis of [^{125}I] EGF binding (*6,8*). EGF iodination was performed by the iodogen method.

Preparation and Characterization of Antibody to Phosphotyrosine

Polyclonal antibody against phosphotyrosine (P-tyr) was prepared in rabbits (New Zealand White) according to reported method (*9*). Ten milligrams of P-tyr were coupled to 10 mg of BSA with 40 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (*10*). Rabbits were injected intradermally at multiple sites each time with 0.5 mg P-tyr-conjugated BSA. The first injection was in Freund's complete adjuvant, followed by four subsequent injections at monthly intervals in Freund's incomplete adjuvant. Characterizations of the binding specificity of the antiserum was done by an ELISA method following a procedure described in ref. (*11*). Briefly, P-tyr was coupled to KLH by glutaraldehyde. The same was done for phosphoserine (P-ser) and phosphothreonine (P-thr). Microtiter plates were coated with conjugated KLH, followed by the addition of serially diluted antiserum. The amount of immunoglobulin retained by the well was determined by the addition of a HRP-conjugated second antibody (goat antirabbit at 30,000-fold dilution) followed by the HRP color reaction.

Standard Conditions of the Solid-Phase Tyrosine Kinase Assay

The standard assay protocol for the EGF receptor tyrosine kinase in a microtiter plate format is as follows. One hundred microliters of G6PDH solution (1 μM dissolved in 20 mM HEPES, pH 7.5) were pipeted into each well of Nunc-Immuno microtiter plates. Noncovalent immobilization of the enzyme on the well surface was allowed to proceed at room temperature for 2 h, after which the plates were washed with 20 mM HEPES, pH 7.5. A second coating procedure, intended to cover the remaining protein-binding sites to reduce background, using 300 μL 0.5% gelatin dissolved in 20 mM HEPES, pH 7.5, containing 0.1% Triton X-100 and 10% glycerol (HTG buffer), was allowed to proceed at room temperature for 2 h. The plates were washed again with HTG buffer. Phosphorylation was then allowed to proceed in the wells in a final volume of 100 μL at room temperature for 60 min. The solution phase of the reaction cocktail contained the following: 0.05 nM of the solubilized EGF receptor preparation (background control was without the receptor), 1 μM EGF (basal phosphorylation was without EGF), 100 μM ATP, 5 mM Mg^{2+}, and 1 mM Mn^{2+}. After the reaction, the wells were washed with HTG buffer, followed by 0.1M sodium phosphate, pH 7.5, containing 0.2M NaCl. The amount of phosphorylated G6PDH on the wells was determined by the polyclonal antibody against phosphotyrosine in an ELISA format. This involves incubating the wells with 100 μL of a 10,000-fold diluted antiserum for 2 h at room temperature. The buffer used for diluting the antiserum was phosphate-buffered saline (PBS), pH 7.5, containing 0.1% gelatin and 0.1% Tween 20. The wells were then washed with PBS containing 0.1% Tween 20 before incubation with the second antibody. The second antibody used was 100 μL of a 30,000-fold diluted goat antirabbit immunoglobulin conjugated with HRP. Incubation was allowed to proceed at room temperature for 1 h, after which the wells were washed with PBS containing 0.1% Tween 20, followed by 0.1M citrate buffer, pH 5.5. Finally, 150 μL of HRP substrate solution (3 mM H_2O_2, 10 mM *o*-phenylenediamine in 0.1M citrate buffer, pH 5.5) were added. Reaction was allowed to proceed at room temperature for 15 min, after which 100 μL 1.5M H_2SO_4 were added to stop the reaction. Absorbance at 492 nm was taken on an ELISA plate reader.

RESULTS AND DISCUSSION

Specificity of Polyclonal Antibody Against P-tyr

As determined by the ELISA method, the polyclonal antibody reacted specifically with P-tyr-conjugated KLH, but not with the other phosphoamino acid-conjugated KLH or with KLH itself (Fig. 1). In fact, polylysines (data not shown) and unphosphorylated G6PDH (described later) did not

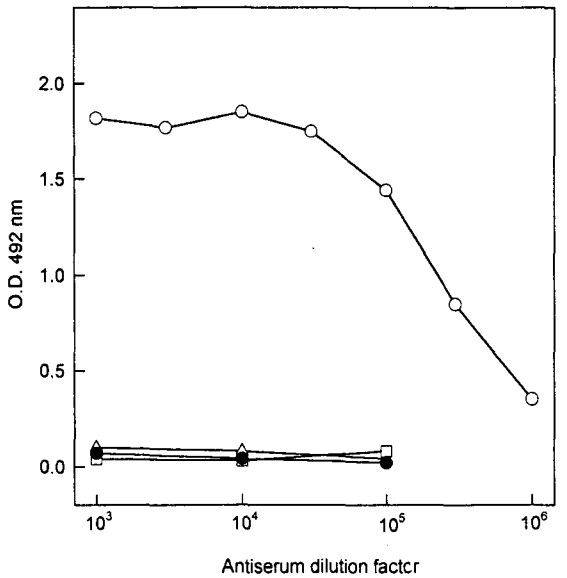

Fig. 1. Specificity of the polyclonal antibody against P-tyr. The rabbit antiserum was characterized for its specificity by an ELISA method as described in Materials and Methods. Microtiter plates were coated with P-tyr-KLH (○), P-ser-KLH (△), P-thr-KLH (□), and KLH (●). Serially diluted serum (dilution factor from 10^3 to 10^6 as indicated on the abscissa) was applied to the wells. The amount of immunoglobulin retained was assayed by adding a goat antirabbit immunoglobulin conjugated with HRP, followed by the HRP reaction. Results are the means of triplicate determinations.

crossreact with the anti-P-tyr antibody. Ten thousand-fold dilution of the antiserum gave a maximum signal, and this dilution of the antiserum was used in subsequent experiments.

Optimization of the Solid-Phase Assay Protocol

Appropriate assay conditions, such as divalent metal ion requirement, ATP concentration, and so forth, have been established for the solution assay of the EGF receptor tyrosine kinase using G6PDH as a substrate (*6*). In the solid-phase assay, these were largely adopted, with the exception that no radioactivity was employed and that immobilized G6PDH (precoated on the well surface of microtiter plates) was used as the substrate, as described in Materials and Methods.

The activity of the EGF receptor tyrosine kinase is stimulated by binding of EGF to the ligand-binding domain of the receptor. The phosphorylation of immobilized G6PDH by solubilized EGF receptor was found to exhibit EGF dependence (Fig. 2). The ED_{50}, corresponding to the value on the abscissa at the inflection point of the curve in Fig. 2, was $1.47 \times 10^{-8}M$, and this is consistent with the affinity of the solubilized receptor for the ligand. Maximal stimulation was observed at $3 \times 10^{-7}M$ EGF or beyond.

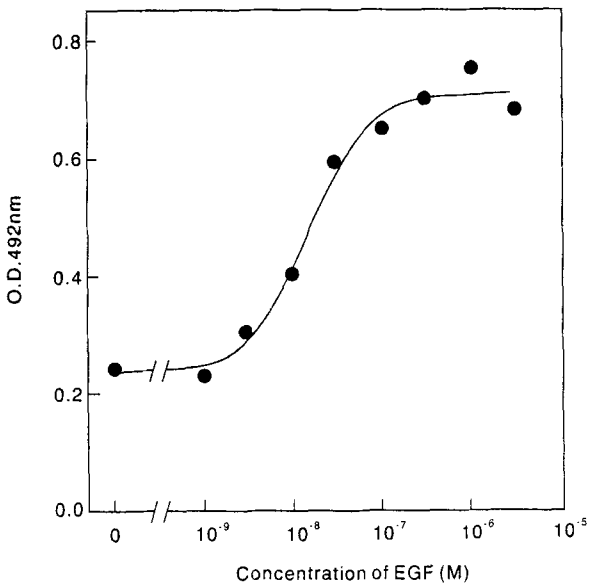

Fig. 2. EGF dependence of G6PDH phosphorylation. The solid-phase receptor kinase assay was performed under the standard conditions described in Materials and Methods, except that different amounts of EGF were incorporated during the phosphorylation step, as indicated on the abscissa. Results are means of triplicate determinations. The curve drawn was obtained from a four-parameter logistic curve fit of the experimental data.

Immobilization of the substrate is the key to the successful development of the solid-phase assay. To this end, we have tried various microtiter plates from different commercial sources. Using our standard protocol, the microtiter plates that gave consistently high signal-to-noise ratio are the Immuno Plates (MaxiSorp type) from Nunc.

Figure 3 shows the effect of varying the G6PDH concentration of the coating solution. No further increase in ELISA signal was detected for concentration beyond 1 μM, indicating that the available binding sites for the substrate had already been saturated. One micromolar of G6PDH was thus used in subsequent plate-coating procedures. An estimation of the protein concentration of G6PDH in solution before and after the immobilization procedure indicated that approx 10% of the substrate was immobilized onto the well surface. A vigorous concentration dependence of the immobilized G6PDH as a substrate could not be ascertained, because we did not have a direct control of the amount of substrate that could be immobilized. In the solution assay, a four- to fivefold increase in V_{max} was observed in the presence of EGF (*6*). In the solid-phase assay, the fold activation in the signal is around 3.

In Fig. 3, the control was the deletion of G6PDH in the coating process, i.e., the assay background, which was found to be very low. Another control was done by coating the wells with 1 μM G6PDH, but the phosphorylation reaction was carried out in the absence of receptor kinase.

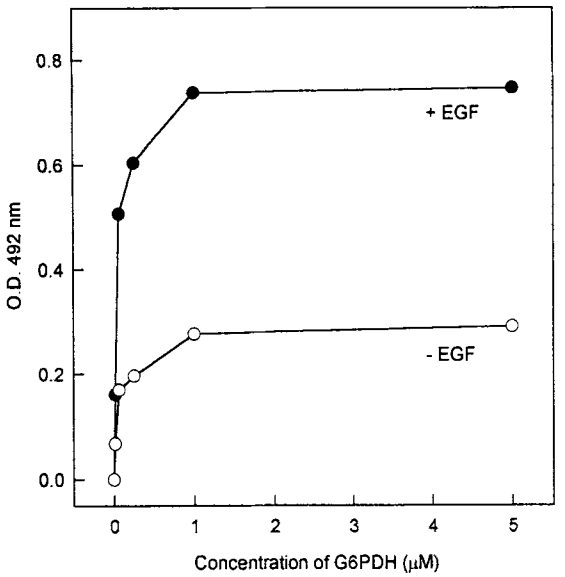

Fig. 3. Saturation curve of substrate binding to the microtiter plate. Microtiter plates were coated with G6PDH solutions of different concentrations as indicated on the abscissa. Phosphorylation reactions were carried out either in the presence (●) or absence (○) of 1 μM EGF. Anti-P-tyr ELISA was then performed under the standard conditions as described in Materials and Methods. Results are the means of triplicate determinations.

Again negligible signal was obtained. This agrees with the antibody specificity studies where it was demonstrated that the antiserum did not recognize unphosphorylated G6PDH.

Before settling down to our standard assay protocol, we have also attempted to precoat the wells with polylysine prior to the G6PDH immobilization process with a view to increasing the amount of substrate bound. Polylysines of different molecular weights (mol wt) and optical activity have been tried (Table 1), namely poly-D-lysine (mol wt 14,000), poly-D-lysine (mol wt 100,000), poly-L-lysine (mol wt 8400), and poly-L-lysine (mol wt 30,000). Polylysine itself did not crossreact with the antibody, but its inclusion appeared to have no beneficial effect to our assay system. In fact, both the activation by EGF and the absolute difference of the OD readings deteriorated on prior coating of the microtiter plates with polylysine (Table 1). The polylysine pretreatment step was therefore not included in the standard protocol.

Overnight coating of microtiter plates with G6PDH solution at 4°C gave equally good results. Noncovalent immobilization is a temperature-dependent process with a slower rate at lower temperatures. Coating of plates at 4°C, though slower, has the advantage that a large number of plates can be processed in a batchwise manner for subsequent experiments. In our laboratory, we have prepared plates with G6PDH solution, properly sealed to prevent evaporation, and stored at 4°C for up to 2 wk

Table 1
Effect of Precoating Microtiter Plates with Polylysine on the Performance of Using Immobilized G6PDH as Substrate for the Solid-Phase Receptor Kinase Assay

Type of polylysine used	NIL	D-14,000			D-100,000			L-8400			L-30,000		
Conc. of polylysine used (μg/mL)	—	100	10	1	100	10	1	100	10	1	100	10	1
−EGF	0.308	0.645	0.658	0.627	0.620	0.669	0.631	0.654	0.649	0.572	0.682	0.720	0.557
+EGF	0.750	0.854	0.870	0.857	0.724	0.749	0.801	0.795	0.798	0.875	0.894	0.853	0.780
+EGF/−EGF	2.44	1.32	1.32	1.37	1.17	1.12	1.27	1.22	1.23	1.53	1.31	1.18	1.40
(+EGF)-(−EGF)	0.442	0.209	0.212	0.230	0.104	0.080	0.170	0.141	0.149	0.303	0.212	0.133	0.223

Polymers of either L-lysine or D-lysine of different molecular weights were precoated on the wells: D-14,000 (poly-D-lysine of mol wt 14,000), D-100,000 (poly-D-lysine of mol wt 100,000), L-8,400 (poly-L-lysine of mol wt 8,400), and L-30,000 (poly-L-lysine of mol wt 30,000). Three different concentrations of polylysine were used in the coating process, as indicated. The control was without the polylysine precoating procedure. Wells were then coated with 1 μM G6PDH, followed by the phosphorylation reaction, either in the presence (+) or absence (−) of 1 μM EGF. Anti-P-tyr ELISA was then performed under the standard conditions as described in Materials and Methods. Values of OD readings at 492 nm were recorded. +EFG/−EGF represents the fold activation of the signal by EGF in the phosphorylation reaction. (+EGF)−(−EGF) represents the absolute difference of the OD readings.

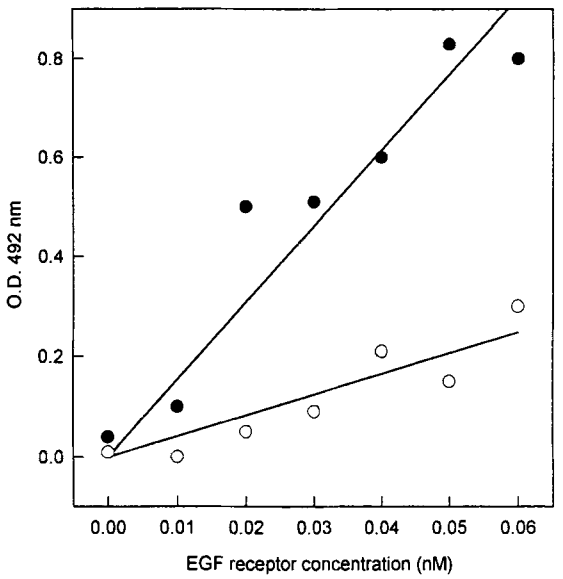

Fig. 4. Receptor kinase concentration dependence of the phosphorylation reaction. The solid-phase receptor kinase assay was performed under the standard conditions described in Materials and Methods, except that different amounts of EGF receptor were used in the phosphorylation reaction, as indicated on the abscissa. Reactions were carried out either in the presence (●) or absence (○) of 1 μM EGF. Results are the means of triplicate determinations. The line drawn was obtained from a linear regression of the experimental data.

without affecting the consistency of the results. The shelf life of the precoated plates can be made longer, say, by incorporation of a low concentration of bactericide. The only consideration is the chemical stability of the protein substrate as well as bacterial growth.

The phosphorylation of the immobilized G6PDH in the solid-phase assay was also shown to be dependent on the concentration of the receptor kinase (Fig. 4). The reaction with respect to both basal phosphorylation and EGF-stimulated phosphorylation appeared to be linear up to 60 or 80 min (Fig. 5).

Receptor tyrosine kinase activity is sometimes assayed by measuring the incorporation of radioactive phosphate into synthetic peptide substrates, such as random polymers containing tyrosine residues (*12*). The suitability of such synthetic peptides to act as substrates in the solid-phase assay was assessed (Table 2). Of the two random tyrosine-containing polymers tested, namely poly (Glu^6, Ala^3, Tyr^1) and poly (Glu^4, Tyr^1), they were found to be inferior to G6PDH as a substrate, even using elevated concentrations for coating. Using the random polymers as substrate, the fold activation by EGF was similar to that of G6PDH. However, the absolute signals were much lower and are therefore much more prone to experimental error (Table 2).

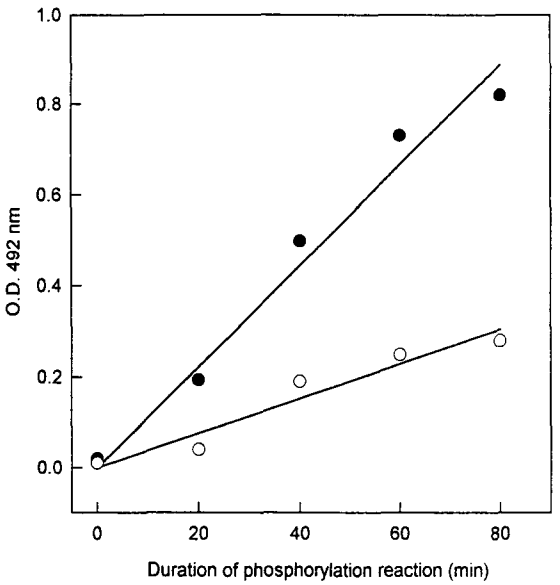

Fig. 5. Time-course of the phosphorylation reaction. The solid-phase receptor kinase assay was performed under the standard conditions described in Materials and Methods, except that the phosphorylation reactions were stopped at different time intervals, as indicated on the abscissa. Reactions were carried out in the presence (●) or absence (○) of 1 μ*M* EGF. Results are the means of triplicate determinations. The line drawn was obtained from a linear regression of the experimental data.

Table 2
Solid-Phase Receptor Kinase Assay: A Comparison of Different Substrates

Type of substrate used	G6PDH	EAY		EY	
Substrate conc. in the coating procedure	1 μ*M*	20 mg/mL (426 μ*M*)	2 mg/mL (42.6 μ*M*)	20 mg/mL (465 μ*M*)	2 mg/mL (46.5 μ*M*)
−EGF	0.284	0.048	0.059	0.058	0.067
+EGF	0.702	0.148	0.146	0.148	0.164
+EGF/−EGF	2.47	3.08	2.47	2.55	2.45
(+EGF)−(−EGF)	0.418	0.100	0.087	0.090	0.097

Microtiter plates were coated with G6PDH or a random polymer containing tyrosine residues. Two such polymers were used, namely EAY: poly (Glu^6, Ala^3, Tyr^1) having an average mol wt of 47,000 and EY: poly (Glu^4, Tyr^1) having an average mol wt of 43,000. One micromolar of G6PDH and two concentrations of the polymers were used in the coating process, as indicated. Phosphorylation reactions were performed either in the presence (+) or absence (−) of 1 μ*M* EGF, followed by anti-P-tyr ELISA as described in Materials and Methods. OD readings at 492 nm were recorded. +EGF/−EGF represents the fold activation of the signal by EGF in the phosphorylation reaction. (+EGF)−(−EGF) represents the absolute difference of the OD readings.

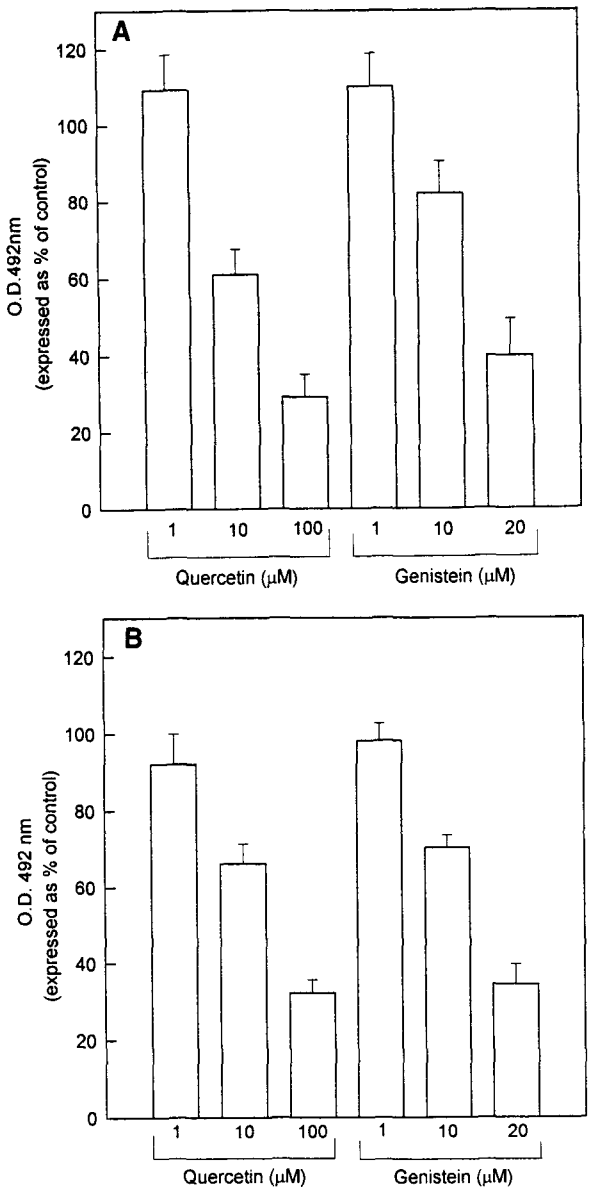

Fig. 6. Effect of kinase inhibitors on the solid-phase receptor kinase assay. The solid-phase receptor kinase assay was performed under the standard conditions described in Materials and Methods. Different amounts of kinase inhibitors were incorporated during the phosphorylation reaction: quercetin (at 1, 10, and 100 μM) and genistein (at 1, 10, and 20 μM). Controls were without inhibitor. (**A**) The basal phosphorylation where the reactions were performed in the absence of EGF. (**B**) The EGF-stimulated phosphorylation where the reactions were performed in the presence of 1 μM EGF. Data are expressed as percentage of control. Results are the means and SEMs of four independent experiments.

The effect of known tyrosine kinase inhibitors on the solid-phase assay was also assessed. Two such inhibitors were used, namely quercetin (*6,13*) and genistein (*14,15*). Different concentrations of these inhibitors were incorporated in the solid-phase assay during the phosphorylation step. Results are presented in Fig. 6. Both quercetin and genistein pro-

duced a dose-dependent inhibition of the EGF receptor tyrosine kinase in the solid-phase assay using immobilized G6PDH as a substrate, in both the basal phosphorylation as well as the EGF-stimulated phosphorylation. Although the results did not permit an accurate determination of the IC_{50} value, it could however be estimated that the IC_{50} for quercetin is around 10 μM. For genistein, the IC_{50} is estimated to be somewhere between 10 and 20 μM. These values are similar in magnitude to that obtained by other assay methods, indicating the validity of the solid-phase assay for screening putative kinase inhibitors.

Quercetin is a flavonoid, genistein is an isoflavone, and both of them are of plant origin. One reason for choosing them as model inhibitors in the solid-phase assay is their similarity in structure to some of the compounds that we are screening for putative tyrosine kinase inhibitory activity. At the moment, we are looking at phytochemicals isolated from Chinese medicinal herbs for kinase inhibitors. Many antitumor agents are derived from natural products (*16*), and it would be interesting to search for novel kinase inhibitors from this huge repertoire. One of the problems confronting us in this endeavor is the large number of compounds that we have to screen. In addition, kinase inhibitory activity is also used to monitor the isolation process. A rapid, convenient, and high-capacity assay system is therefore of paramount importance. We are currently using the solid-phase assay system for this purpose, the results of which will be presented later.

CONCLUSION

This article describes the use of immobilized G6PDH as a substrate for solubilized EGF receptor tyrosine kinase. This solid-phase assay system for tyrosine kinase activity largely alleviates drawbacks of the solution assay, namely, the involvement of radioactivity and a cumbersome experimental procedure are eliminated. The target of arriving at an assay protocol that is simpler, more convenient, and therefore more amenable to automation for large-scale assays is achieved.

Our own criticism of this method is its semiquantitative nature. In the solution assay, the kinetics of the solubilized receptor kinase toward the soluble G6PDH as a substrate were carefully worked out (*6*). In the solid-phase assay in its present form, a full understanding of the kinetics of the solubilized receptor kinase toward the immobilized G6PDH as a substrate is lacking. This is owing, in part at least, to the fact that the amount of G6PDH that can be coated on the well surface of the microtiter plates, an important factor in determining the signal-to-noise ratio in our assay, cannot be entirely controlled. Notwithstanding this, the greatest advantage of the present protocol is the capacity to handle a large number of assays at the same time. This will have immense application potentials in, say, drug-screening programs where a large number of compounds can be

massively screened for tyrosine kinase inhibitory activity. The lead compounds can then be singled out for more vigorous investigations.

In addition, there is no reason why the general methodology of the present protocol cannot be applied to other soluble or solubilized tyrosine kinase systems using another appropriate protein substrate, as long as the substrate can be immobilized on the well surface of the microtiter plate to an adequate level. Taking this further, the enzyme to be assayed might not even be a tyrosine kinase or a kinase at all, as long as an antibody is available that can detect a specific feature of the product converted from the immobilized substrate.

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